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New Findings Section

What is the central question of this study?

Although peripheral blood HPCs are potentially important in regeneration after acute myocardial infarction (MI), their self-renewal ability in the post-acute phase has not yet been addressed.

What is the main finding and its importance?

In rat peripheral blood, we show that MI does not negatively affect circulating HPC self-renewal ability two weeks after acute infarction which suggests a constant regenerative potential in the MI post-acute phase.

Abstract

Due to the importance of peripheral blood hematopoietic stem and progenitor cells (HPCs) in post-acute regeneration after acute myocardial infarction (MI), the present study aimed to investigate count and secondary replating capacity/self-renewal ability of HPCs in peripheral blood before and two weeks after MI. In nine female Lewis inbred rats (n=9), myocardial infarction was induced by ligation of the left coronary artery while another nine had surgery without ligation for control purposes. Myocardial infarction was confirmed by troponin I concentrations 24h after MI. Peripheral blood was withdrawn and fractional shortening (FS) and ejection fraction (EF) of the left ventricle was assessed before (day-0) and 14 days after MI or control surgery (day-14). After mononuclear cell isolation, primary/secondary functional colony-forming unit-granulocyte macrophage (CFU-GM) assays were performed in order to detect the kinetics of functional HPC colony count and cell self-renewal ability *in vitro*. CFU-GM count and cell self-renewal ability remained unchanged ($p>0.05$) in both groups at day-14 without interaction between groups. In the intervention group, higher day-0 CFU-GM counts showed a relation to lower FS on day-14 ($\rho=-0.82$; $p<0.01$). Myocardial infarction did not negatively

affect circulating HPC self-renewal ability, which suggests a constant regenerative potential in the post-acute phase. A relation of cardiac contractile function 14 days after MI with circulating CFU-GM count on day-0 might imply functional colony count as a predictive factor for outcome after infarction.

Introduction

Acute myocardial infarction (MI) is characterized by a generalized inflammatory reaction triggering the rapid mobilization of stem and progenitor cells from bone-marrow (BM), which are important for acute and post-acute regeneration processes. The contribution of circulating cells to myocardial repair, however, is not yet fully understood, although experimental studies have seen the potential in bone-marrow derived precursor cells for cardiac regeneration after MI(Wojakowski *et al.*, 2012). The possible therapeutic effect of circulating hematopoietic stem and progenitor cells (HPCs) on damaged heart tissue is currently the subject of intense debate(Anversa *et al.*, 2013). The current predominant view is that these cells secrete a variety of cytokines that activate endogenous progenitors in the heart muscle, which are responsible for the repair process and the improvement in e.g. ventricular function(Anversa *et al.*, 2013). Therefore the body's own regenerative ability (to counteract pathological heart remodeling and improve ejection fraction/systolic function) depends, among others, on the circulating number and secondary replating capacity/self-renewal ability of these cells after the infarction. Although some human as well as animal studies have documented the kinetics of HPC in the first week after infarction(Paczkowska *et al.*, 2005; Assmus *et al.*, 2012; Wojakowski *et al.*, 2012), the post-acute phase (e.g. 14 days after MI) has had little attention so far while long-term outcomes mainly assessed endothelial progenitor cell kinetics(Regueiro *et al.*, 2015). Acute myocardial infarction was shown to increase the number of immune cells(Tsujioka *et al.*, 2009; Gentek & Hoeffel, 2017), BM(Assmus *et al.*, 2012) and peripheral blood (PB)(Leone *et al.*, 2005; Assmus *et al.*, 2012) HPCs one week after MI with BM functional HPC colony number being

elevated after MI, possibly due to an increased BM activity. Peripheral blood HPCs stayed elevated two weeks after MI in a rat model(Abdelmonem *et al.*, 2015), but the self-renewal ability of PB HPCs was not assessed, although this would provide important information regarding self-regeneration during this post-acute phase. In addition, controversy exists as to whether the initial increase in HPC number would have declined towards baseline by day-14 as extrapolated from literature results(Shintani *et al.*, 2001). Therefore, the aim of the present study was to investigate i) the functional colony count and self-renewal ability of circulating HPCs in the peripheral blood and their association with heart function and ii) the MI-induced change of risk-associated blood cell counts, e.g. neutrophil-to-lymphocyte ratio, in a rat model.

Methods

Ethical approval

All animals received care in compliance with the European Convention on Animal Care. The surgical procedures were performed in accordance with the Swiss Animal Protection Law after obtaining permission from the State Veterinary Office, Fribourg, and approval from the Swiss Federal Veterinary Office, Switzerland (Ethics approval number: 2013_09E_FR). All procedures used conform to the principles and regulations as described by given guidelines(Grundy, 2015).

Animals, invasive procedures and post-operative care

Eighteen female LEWIS inbred rats (weight=215.2±11.3g; from Janvier, France) were divided into an intervention group (IG, n=9) and a control group (CG, n=9). Food (normal diet/pellets) and fresh water was provided ad libitum. One rat of the CG unexpectedly died before day-14.

As previously described(Frobert *et al.*, 2014), animals were placed in an induction chamber with 5% isoflurane in oxygen for 5-7 min. A toe and tail pinch confirmed sedation. Animals were then placed on a warming pad at 37°C and intubated. Anesthesia was sustained with 2.5% isoflurane in oxygen at

a minute ventilation of 2.5 L/min. Depth of anesthesia was continuously monitored by assessment of tail-pinch reflex and respiratory rhythm. Following a left thoracotomy between the fourth and fifth interstitial space, the pericardium was opened to access the heart and the left anterior descending coronary artery (LAD) was ligated only for animals of the IG, while animals of the CG were closed without ligation. The investigators took all possible steps to minimize animals' pain and suffering. One third of the dose of buprenorphine (0.1 mg/kg) was injected subcutaneously 20-30 min before the beginning of the anesthesia and two third immediately before the end of the surgery as post-operative analgesia. A second dose was administered 6h after surgery and buprenorphine was added to the water during the night. Further doses were administered after 24h and 48h with supplementation of the water with buprenorphine during the night. Between 24 and 48h, further doses of analgesics were administered depending on the score during post-operative pain evaluation. Welfare monitoring of animals via behavioral observation was performed every day. After surgery, the animals were kept separately in a cage warmed with a heat lamp until they had fully recovered from anesthesia. They were then put back together, 5 rats per cage (1800 cm^2), according to the Eurostandard type III. Food (pellets) and fresh water was provided ad libitum. A 12h:12h light:dark cycle and a constant temperature of 24°C were maintained during the entire experimentation time. Blood sampling was performed under general anesthesia maintained with 2.5% isoflurane in 2.5L/min oxygen with a face mask. At the end of the study, animals were humanely killed. Exsanguination was performed under general anesthesia (isoflurane 2.5% in 2.5L/min oxygen), the thorax was opened, blood withdrawn from the vena cava and the heart harvested.

To verify infarct development (IG only), a blood sample was collected from the caudal tail artery at 24h post LAD ligation. Plasma was stored at 80°C. Troponin I quantification was performed as previously described(Frobert *et al.*, 2015) using the AccuTNI3+ immunoassay. Heart function was assessed under light anesthesia (2% isoflurane) using a Vevo3100 (Visualsonic) high-resolution ultrasound imaging system. Pre-MI (day-0), 24h (day-1), 1 week (day-7) and 2 weeks (day-14) post

MI, the ejection fraction (EF) was determined in B-Mode and the fractional shortening (FS) in M-Mode on a parasternal long axis view.

Mononuclear cell isolation

At baseline and 14 days after MI induction or control surgery, peripheral blood was withdrawn from the caudal vein (700-1000µL) into lithium-heparinized tubes of which 100µl of whole blood was kept for hematological analysis and the remaining volume was subjected to a standard Ficoll gradient centrifugation (Histopaque; Sigma-Aldrich) according to the manufacturer's instruction in order to isolate peripheral blood mononuclear cells for hematopoietic stem and progenitor cell functionality tests such as functional colony number and secondary replating capacity/self-renewal ability (primary, secondary colony-forming unit assays respectively).

Analysis of blood cell counts

Blood cell counts were analyzed at baseline (day-0) and 14 days after MI or control surgery (day-14) using 100µL of whole blood. Analysis was performed with a general rat program of a hematology analyzer (dilution 1:3, 1:6 or 1:12, ADVIA 2120i, Siemens, Zurich, Switzerland). Lithium-heparin anti-coagulated blood has some limitations for blood cell analysis (e.g. platelet clumping(Guzman *et al.*, 2008)), which was taken into account for the presentation of final results.

Primary/Secondary colony-forming unit assays

Primary and secondary hematopoietic colony-forming unit granulocyte, macrophage (CFU-GM) assays were performed as published(Stelzer *et al.*, 2010) with slight modification of the procedure for peripheral blood. Peripheral blood mononuclear cells were plated at a concentration of 200'000 cells/mL in methylcellulose culture medium for rats (Methocult GF R3774; StemCell Technologies, Vancouver, Canada) in 12-well flat-bottom plates and incubated at 37°C (5% CO_2, >95% humidity). On the 6th day of incubation, colonies consisting of at least 50 cells were scored providing the number of functional hematopoietic progenitor cells (primary CFU-GM) of each animal. Colonies were

counted and up to 60 primary CFU-GM colonies were individually plucked from the methylcellulose culture medium and transferred to individual wells of a 48-well flat-bottom plate and thoroughly mixed with methylcellulose culture medium to get a single-cell suspension. After another 8 days of incubation, each well was scored for the number of CFU-GM colonies consisting of more than 50 cells (secondary CFU-GM). The secondary replating capacity is known to be associated with the proliferative capacity of myeloid progenitor cells(Gordon *et al.*, 1998; Withey *et al.*, 2005). The original protocol for human cells was modified for rat peripheral blood by transferring fewer colonies (up to 60 instead of 90), since rat cells have a higher CFU-GM replating efficiency than human cells with respect to the number of clonogenic primary colonies and the produced number of secondary colonies per primary replated CFU-GM(Alenzi *et al.*, 2002).

Analysis of secondary CFU-GM assays

For analysis of secondary CFU-GM assays, the number of secondary CFU-GM produced by each primary CFU-GM was used as raw data. Counts exceeding 100 were truncated. The secondary replating capacity of an individual was defined to be the mean of the log 2 of the number of colonies plus one for the following reasons: The log 2 scale is natural, as the distribution of number of secondary colony-forming cells is skewed to the right. One was added, as the log 2 of 0 cannot be calculated and the log 2 of this number is a continuous measure of the number of duplications of a primary CFU-GM. This measure has similar properties as the measure used by Gordon et al. (1998)(Gordon *et al.*, 1998) as counts of zero are adequately taken into account and the log scale reduces skewness.

Statistics

Data are represented as mean ± standard deviation (SD) unless otherwise stated. A-priori power analysis (paired t-test) was used to calculate the necessary sample size. Based on published differences(Paczkowska *et al.*, 2005; Assmus *et al.*, 2012; Wojakowski *et al.*, 2012) in CFU-GM

bone-marrow colony number before (45±15) and 7 days after MI (69±18) in mice, an alpha error of 0.05, a 1-beta error of 0.8, and an effect size of 1.437, a sample size of n=6 was determined. To consider possible drop-outs, a total sample size of n=9 was chosen. A two-way ANOVA was carried out in order to compare the time effects between day-0 and day-14 and possible interactions among groups. Post-hoc tests were performed when appropriate. Pearson's correlation analysis was used to determine the relationship between variables.

Results

Confirmation of infarction

Cardiac troponin I (cTnI) was significantly increased 24h after MI (26.01±6.08 ng/mL) in the IG. Fractional shortening and EF were significantly reduced already after 24h and stayed low until 2 weeks post MI (Fig 1A, B).

Progenitor cell number and secondary replating capacity/self-renewal ability

At baseline, groups were comparable ($p>0.05$) regarding both CFU-GM number and cell self-renewal ability. Two weeks after MI, on day-14, both CFU-GM number and HPCs' self-renewal ability did not differ from respective baseline values ($p>0.05$, Fig 2A, B). There was no statistical interaction between groups ($p>0.05$). Also, individual changes in CFU-GM number were not related to respective changes in self-renewal ability. In the IG, CFU-GM number on day-0 and FS on day-14 were significantly associated ($\rho=-0.82$; $p<0.01$, Fig 3). Cell self-renewal ability on day-0 was not associated with cardiac function on day-14 ($p>0.05$).

Blood cell counts

Time effects for blood cell counts (Table 1) of the IG showed significant increases of mean corpuscular volume (MCV, $p<0.001$), monocytes (MONO, $p<0.05$), neutrophils (NEU, $p<0.01$), basophiles (BASO, $p<0.05$) as well as percent lymphocytes (percLYM, $p<0.01$), monocytes (percMONO, $p<0.05$), neutrophils (percNEU, $p<0.01$), basophiles (percBASO, $p<0.05$) and neutrophil/lymphocyte (NL) ratio ($p<0.01$) two weeks after MI. In the CG only MONO, and percMONO (both $p<0.01$) were significantly elevated at day-14 after surgery. Only percLYM, percNEU and NL ratio showed a significant interaction between groups ($p<0.05$).

Posthoc tests between groups revealed that the CG and the IG were comparable at baseline for all variables, except for the IG having higher percNEU values (both $p<0.01$). At day-14 the groups differed in percNEU, percLYM (both $p<0.05$) and also NL ratio ($p<0.05$) with percNEU and NL ratio being higher and percLYM lower in the IG.

Day-0 FS was significantly correlated with day-0 MCV ($r=-0.709$, $p<0.05$), while day-14 FS correlated significantly with percMONO ($r=0.738$, $p<0.05$).

Discussion

This study investigated the regenerative potential of circulating HPCs in the post-acute phase after MI in a rat model. Interestingly, no significant change in mean functional progenitor cell number (CFU-GM count) and cells' self-renewal ability was present in the IG nor the CG 14 days after induction of MI or control surgery, although ejection fraction and fractional shortening were significantly reduced (Fig 1A, B; Fig 2A, B) in the IG.

Our results extend the current knowledge as we assessed the functional count of HPCs as CFU-GM in cell culture rather than CD34+ cell number. Wojakowski et al. (2004), for example, reported circulating CD34+ cell number being elevated one week after MI in comparison to healthy control subjects(Wojakowski *et al.*, 2004). A similar finding was reported in a rat model with induced MI

where CD34+ cell number one week after MI was compared to baseline prior to MI(Lehrke *et al.*, 2006). It was also shown that CD34+ cell count was highest 7 days after MI in humans and was lower on day 14, reaching values comparable to the control group(Shintani *et al.*, 2001). This is consistent with our results in the rat model. In addition, literature states that circulating progenitor cells should be back to baseline levels already 24h after an operative procedure(Choi *et al.*, 2010).

We also showed that the HPCs' self-renewal ability in the post-acute phase after MI on average was not reduced compared to baseline and showed the same behavior as in the CG. Our results therefore imply that HPCs circulating in the post-acute phase after MI are still as functionally competent as before MI and still have the potency to form colonies. Unfortunately, previous studies reporting increased BM and PB HPCs/CFU-GMs up to one or two weeks(Leone *et al.*, 2005; Assmus *et al.*, 2012; Abdelmonem *et al.*, 2015) after MI, did not investigate the self-renewal ability of PB HPCs, such that our results cannot be compared to these(Xin *et al.*, 2008).

Looking at Fig 2B more in detail, it is visible that three animals of the IG showed an increased self-renewal ability, while the other six animals had constant to decreasing values. This is an interesting observation, since these three could resemble the picture of progenitor mobilization associated with a higher *ex-vivo* expansion of CD34+ cells(Ivanovic *et al.*, 2010) after MI showing survival of only the "fittest" progenitor cells with the highest self-renewal ability.

The presence of an on average constant regenerative potential of HPCs in the post-acute phase supports the body's active self-regenerative potential although the IG in comparison to the CG showed signs of MI-induced inflammation such as increased percNEU- associated with increased long-term mortality in acute MI patients(Gentek & Hoeffel, 2017)- and NL ratio(Caimi *et al.*, 2016) two weeks after MI onset. The decreased percLYM at day-14 in the IG might be due to the post-infarction cardiac impairment that triggered important autonomic reflexes (e.g. sympathetic overdrive) and could also impact lymphocytes' physiology in a nonclassical fashion(Nunes-Silva *et al.*, 2017). Within groups, the significant increase in mature neutrophil and monocyte numbers, but unchanged

differentiation of immature HPC (CFU-GM number) could imply disease(Gentek & Hoeffel, 2017) or non-disease(Selig & Nothdurft, 1995) related inflammation. Furthermore, it is important to consider that an automatic hematology analysis system, such as ADVIA 2120i, only distinguishes monocytes and neutrophils according to their morphology- e.g. levels of peroxidase activity(Canovi & Campioli, 2016)- but not based on their functionality. Immature cells being functionally competent to form colonies under *in vitro* conditions might not show the same dynamics as the different mature myeloid subgroups or total white blood cell counts found with flow cytometry.

Day-0 functional CFU-GM count was negatively correlated with FS 14 days after MI (Fig 3), which might indicate a predictive potential of the baseline colony number for the contractile function in the post-acute phase after MI. A higher number of angiogenic colony forming units (CFU-A) was already found to be significantly associated with cardiovascular disease risk(Mavromatis *et al.*, 2012), but no study has yet reported the possible prediction of MI outcome by pre MI CFU-GM colony number. Results of our study extend these findings of Mavromatis et al. (2012), where a higher circulating proangiogenic cell activity by CFU-A was associated with worse clinical outcome in those with CVD. Functional CFU-GM count of circulating HPCs can be suggested to have a predictive potential in a pre-diseased state, as it had already been suggested for CD34+ count and the prediction of future metabolic deterioration in healthy individuals(Fadini *et al.*, 2015). This would mean that constant tissue-regeneration and substitution in the healthy come along with a comparatively low circulating CFU-GM number and a “better” outcome after a deteriorating cardiovascular incidence such as MI.

Interestingly- besides significantly increased cTnI concentrations 24h after LAD ligation- our high-resolution ultrasound imaging system showed a significant reduction of the heart function parameters FS (M-mode) and EF (B-mode) already. Cardiac troponin I levels provide an excellent quality control of infarct size and may be used as a prognostic marker(Frobert *et al.*, 2015). High-resolution microimaging has already been shown to be a useful method for the accurate assessment of cardiac

function in mice(Okajima *et al.*, 2007) and with this study was first proved to be adaptable to a rat model. The drop in heart function for FS and EF remained low until day-14 post MI.

In our study FS was related to percMONO on day-14. Distinct MONO subsets have already been suggested to predict cardiovascular events in heart-disease patients(Rogacev *et al.*, 2012), but the relationship between MONO and cardiac function two weeks post MI has not yet been found. Our result could indicate that the relative amount of circulating monocytes is a possible indicator for cardiac contractile function.

In conclusion, myocardial infarction did not negatively affect circulating HPC self-renewal ability on day-14 after MI, which suggests a constant regenerative potential in the post-acute phase after MI. Possibly, day-0 circulating CFU-GM count might have the potential to predict outcome after infarction which would stress the importance of interventions able to support life-long regeneration in the (still) healthy such as regular physical exercise.

Limitations

One limitation could be seen in the baseline difference of perNEU counts. This difference might be due to IG and CG being from the same brand of rats but coming from different litters. To minimize bias, the same animals were used at baseline and after two weeks. Therefore, peripheral blood was investigated instead of bone marrow. Ideally cell proliferation would have been measured by Ki-67/PI staining(Kim & Sederstrom, 2015) using flow cytometry in parallel to the cell culture experiment. This, however, was not possible due to the limited sample material available.

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Additional information

Competing interests

All authors do not state any competing interests.

Author contributions

The experiments were performed partly at the laboratory space of the Cardiology Group (University of Fribourg, Switzerland) and the Division of Hematology (University Hospital Zurich, Switzerland). JK, CMS, AF and MNG designed the work, took part in acquisition, analysis, or interpretation of data for the work and drafted or revised it critically for important intellectual content. GA took part in data acquisition and analysis and revised the work critically for important intellectual content. All authors approved the final version of the manuscript, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved and qualified for authorship. All those who qualified for authorship were listed.

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Table 1	CONTROL GROUP			INTERVENTION GROUP		
Blood Collection	*Day-0*	*Day-14 post MI*	*N*	*Day-0*	*Day-14 post MI*	*N*
WBC, $10^9/L$	7.90 ± 1.55	9.91 ± 4.05	6	8.72 ± 1.12	10.37 ± 1.59	9
RBC, $10^{12}/L$	8.1 ± 0.9	7.9 ± 1.3	6	8.2 ± 0.6	7.5 ± 2.0	8
Hct, *%*	39 ± 5	39 ± 7	6	41 ± 4	39 ± 10	8
Hgb, *g/dL*	15.8 ± 1.6	24.9 ± 26.0	6	15.7 ± 1.7	14.9 ± 4.6	8
MCV, *fL*	50.8 ± 2.5	50.6 ± 1.1	6	49.5 ± 1.3	52.3 ± 0.9***	9
Lymphocytes, $10^3/\mu l$	6.58 ± 1.94	7.98 ± 2.76	6	7.41 ± 0.94	7.26 ± 1.02	9
Monocytes, $10^3/\mu l$	0.09 ± 0.07	0.26 ± 0.13**	6	0.09 ± 0.03	0.19 ± 0.12*	9
Neutrophils, $10^3/\mu l$	0.78 ± 0.19	1.6 ± 1.4	6	1.1 ± 0.2	2.8 ± 1.2**	9
Basophils, $10^3/\mu l$	0.02 ± 0.02	0.080 ± 0.102	6	0.003 ± 0.010	0.04 ± 0.03*	9
Lymphocytes, *%*	86.1 ± 0.9	82.4 ± 8.2†	6	85.1 ± 1.3	70.7 ± 9.7**	9
Monocytes, *%*	1.2 ± 0.7	2.4 ± 0.7**	6	1.1 ± 0.3	1.8 ± 1.0*	9
Neutrophils, *%*	10.6 ± 1.2††	13.8 ± 7.3†	6	12.9 ± 1.1	26.4 ± 8.7**	9
Basophils, *%*	0.32 ± 0.17	0.63 ± 0.64	6	0.14 ± 0.07	0.39 ± 0.21*	9
NL ratio	0.126 ± 0.048	0.177 ± 0.122†	6	0.151 ± 0.014	0.396 ± 0.191**	9

Values are means ± SD. WBC, white blood cell count; RBC, red blood cell count; Hct, hematocrit; Hgb, hemoglobin; MCV, mean corpuscular volume; NL ratio, neutrophil-to-lymphocyte ratio; Significant differences between time points are indicated as: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; significant differences to the intervention group for the same time points are indicated as: $^{††}p < 0.01$, $^{†}p < 0.05$

Figure Legends

Fig 1: Fractional shortening (A) and ejection fraction (B) at baseline (day-0), 24h (day-1), one week (day-7) and two weeks (day-14) after induction of acute myocardial infarction (MI) in rats (n=9, mean±SD). It is clearly visible that both parameters show a highly significant decrease already after one day indicating a huge impairment of systolic heart function due to MI.

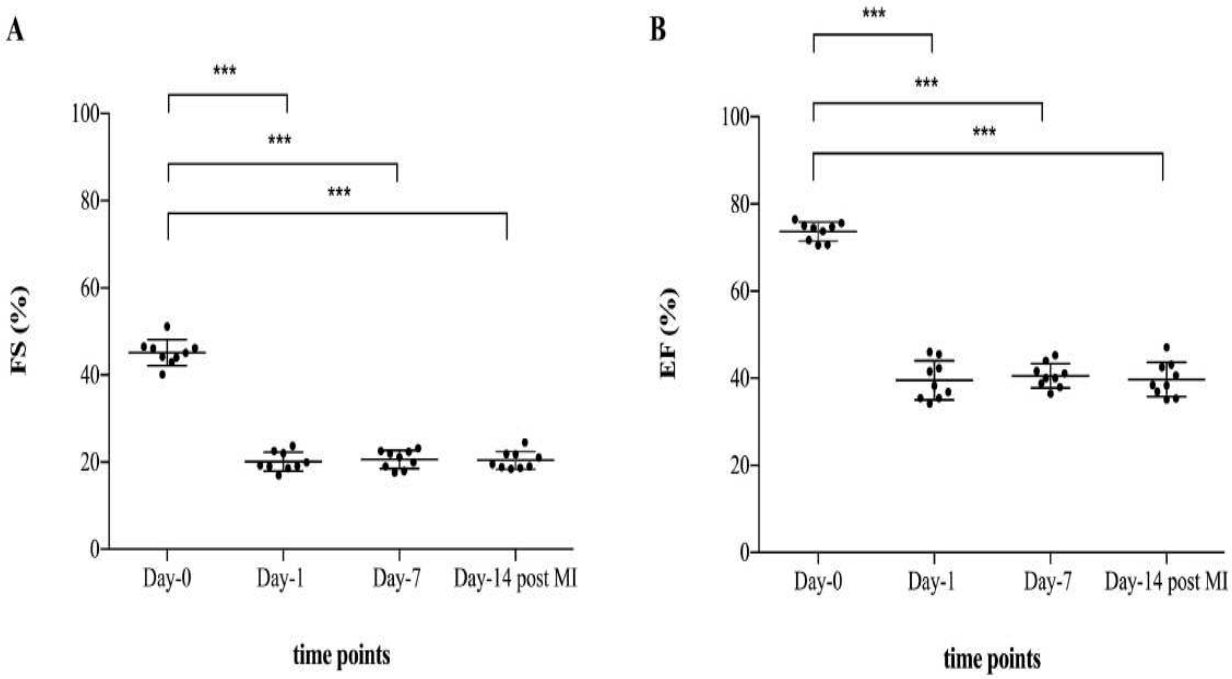

Fig 2: Colony-forming unit granulocyte macrophage (CFU-GM) count per totally plated mononuclear cells (MNC) (A) and HPC's self-renewal ability (B) at baseline (day-0) and two weeks (day-14) after acute myocardial infarction (MI). Parameters did not show any significant change at day-14 nor interaction between groups, which suggests a constant regenerative potential in the post-acute phase after MI.

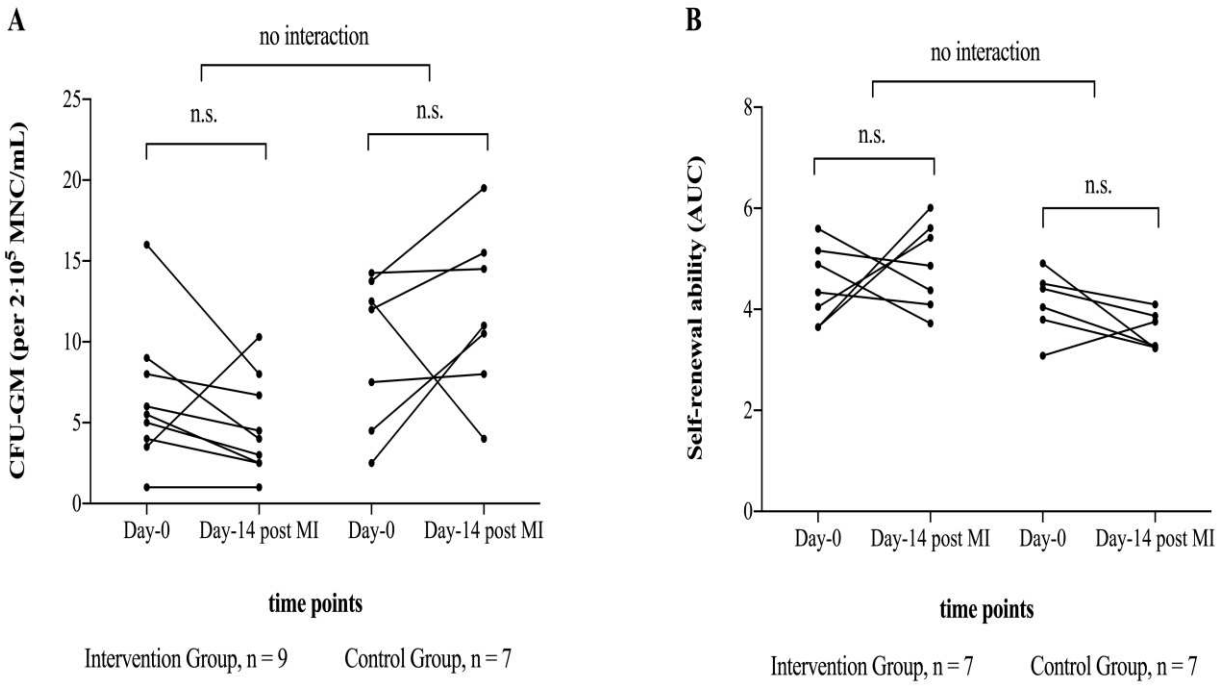

Fig 3: Colony-forming unit granulocyte macrophage (CFU-GM) number at baseline (day-0) and fractional shortening (FS) at two weeks (day-14) post-acute myocardial infarction (MI) were negatively correlated (n=9), which might indicate a predictive potential of baseline colony number for contractile function in the post-acute phase after MI.